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EXPERIMENTAL STUDY

Antimicrobial effect of sodium houttuyfonate on Staphylococcus epidermidis and Candida albicans biofilms

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Abstract

OBJECTIVE: To study antimicrobial effect of Sodium houttuyfonate (SH) on Staphylococcus epidermidis (SE) and Candida albicans (CA).

METHODS: The prepared strain broths (OD_{600}=0.05) containing SE and CA were firstly used to test the minimal inhibitory concentrations (MICs) of SH, azithromycin (AZM) and fluconazole (FLU) by micro-dilution method. Then the biofilms of SE and CA were matured in 96-well plates, and co-cultured with SH, AZM and FLU for 1, 2 and 3 days to assess the antibiofilm efficacies of the agents with different concentrations by crystal violet staining method. At last, the treated biofilms of SE and CA by 2× MIC agents were observed by scanning electronic microscope.

RESULTS: The MICs of SE and CA were 256 and 1024 μg/mL, respectively. After the 1st, 2nd and 3rd day of medications, the suppressions of biofilm were about 60% (P<0.01), 76% (P=0.000) and 75% (P=0.000) by 2×MIC SH, the suppressions of biofilm were about 90% (P=0.000), 88% (P=0.000) and 90% (P=0.000) by 2×MIC SH, which could be testified by scanning electron microscope results. However, the inhibitions of biofilm attachment had no significant difference for SE by SH and azithromycin and CA by SH and fluconazole.

CONCLUSION: SH had widely anti-pathogenic effect on pathogenic biofilm formation of either bacteria or fungus, had more influence on enclosed cells of SE and CA than the traditional antibiotics, revealing its target might be the extracellular polymeric substances, and was more active to inhibit the growth of CA than SE.

Key words: Biofilms; Candida albicans; Drugs, Chinese herbal; Sodium houttuyfonate; Staphylococcus epidermidis

INTRODUCTION

Pathogenic tolerance has become an intractable problem for clinicians and microbiologists in relevant fields due to the recalcitrant biofilm that is mainly composed of extracellular polymer substances to provide protection for enclosed pathogens from host immune system and antimicrobial agents.[1] Traditional antibiotics, such as azithromycin (AZM) for Pseudomonas aeruginosa (PA), vancomycin (VA) for Staphylococcus aureus (SA) and Staphylococcus epidermidis (SE), fluconazole (FLU) and amphotericin B for Candida albicans (CA),

etc., have caused widespread bacterial resistance, and the toxicity will be considerable if large dose is applied.[2-6] Therefore, it is of interest to search alternatives, which can both have decent antimicrobial efficacies or enhance pathogen-killings in combination with traditional antibiotics, and present lower toxicities whenever with relative large dose or long treatment.

Some traditional Chinese herbs or their active extractions, such as baicalin and tetrandrine, exhibited quite good antibacterial effects.[7,8] Sodium houttuyfonate (SH, $CH_3(CH_2)_8COCH_2CHOHSO_3Na$) is a derivative of houttuynin (i.e., decanoyl acetaldehyde, $CH_3(CH_2)_8COCH_2CHO$) from Yuxingcao (*Herba Houttyniae cordatae*) (Saururaceae family) with low toxicity and other clinical effects on ventricular remodeling, myocardial hypertrophy, and bacterial cell membranes, etc.[9-12] According to our previous study, SH alone had mild suppression and enhanced inhibition in combination with levofloxacin on PA biofilm formation.[13]

However, there have been fewer reports on antimicrobial potential and applicability of SH for other pathogens, especially eukaryotic microorganism, such as CA. In this paper, we will focus on the antimicrobial feasibility of SH alone for the suppressions of pathogenic attachments and biofilm mass of SE and CA.

MATERIALS AND METHODS

Preparations of SE and CA

SE ATCC35984 and CA SC5314 were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and the Second Military Medical University (Shanghai, China), respectively. SE and CA were respectively inoculated in Luria-Bertani (LB) broth and Sabouraud medium (Aoboxing Bio-tech, Beijing, China) in constant-temperature shaker GLY (Fuma, Shanghai, China) of 220 r/min at 37℃. After 6 h proliferation for SE and 12 h for CA, the strain was harvested by GL-20G-II high-speed refrigerated centrifuge (Fuma, Shanghai, China) at 2000 r/min for 10 min. The supernate was discarded, and the precipitate was resuspended in pH 7.4 PBS (Golden-bridge, Beijing, China) for absorbance detection at 600 nm (OD_{600}) in UV-spectrophotometer (SHIMADZU, Kyoto, Japan). The optical density was adjusted to 0.05 for use.

Minimal inhibitory concentrations (MICs) test

Microdilution method was adopted to test the MICs of SH, AZM and FLU obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). A gradient of concentrations of the three agents, i.e. 2048, 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 μg/mL were added into 96-well plates with eight wells in duplicate for each gradient, and each well had 100 μL of the three agents and 100 μL inoculum with the final concentration of 1×10^6 cfu/mL. After mixing, the plates with SE and CA were under stationary culture for 24 and 48 h at 37℃ respectively, and then the cells from SE and CA were also inoculated on nutrient agar medium for respective 24 and 48 h at 37℃ to determine the MIC. Each MIC assay was performed in triplicate.[14]

Anti-biofilm by antimicrobial agent alone

(a) The concentrations of antimicrobial agent: The biofilm formations of SE were investigated by SH and AZM, the concentrations of which were respectively set as 1/32×MIC, 1/16×MIC, 1/8×MIC, 1/4×MIC, 1/2×MIC, 1×MIC and 2×MIC. The biofilm formation of CA was investigated by SH and FLU, the concentrations of which were the same as those of SE. The one without agent was set as negative control. (b) Biofilm growing: each well was added by 200 μL pathogenic suspensions for 24 h incubation at 37℃. Then the solution was discarded, and the plate was rinsed by PBS to wash planktonic cells away. Trypticase soy broth (TSB) medium containing corresponding concentrations of antimicrobial agents described above was instilled in each well. The negative control was changed by new TSB medium with no agents. The mediums with and without agents were renewed per day. After 1st, 2nd and 3rd medications, PBS was used to wash out planktonic cells in each well. (c) Biofilm staining: the rinsed plate was added by 125 μL solution containing 0.1% crystal violet (CV) to each well, and incubated at room temperature for 10-15 min. Then, the plate was rinsed 3-4 times with PBS to get rid of excess dye. (d) Biofilm quantifying: The stained plate described above was added by 125 μL 30 % acetic acid in water to each well to solubilize crystal violet (CV), and incubated at room temperature for 10-15 min. Then, 125 μL solubilized CV was transferred to a new microtiter dish to detect absorbance in a stat fax-2100 plate reader (AWARENESS, Burlington, VT, USA) at 490 nm. 30% acetic acid in water was set as the blank.[15] Each detection was performed in triplicate.

Cellular attachment

Autoclaved membrane (0.22 μm, Millipore, Billerica, MA, USA) was placed into 6-well sterile plate with four wells in duplicate for each pathogen. 2 mL TSB broth, 200 μL SE and 200 μL CA suspension (≈1×10^5 cfu/mL) were added in each well for respective 24 and 48 h at 37℃. Then the solution was discarded, the membrane was rinsed by PBS fully and re-placed into the plate. Each well was then given 2 mL medium with antimicrobial agent concentrations of 2×MIC SH and 2×MIC AZM for SE, and 2×MIC SH and 2×MIC FLU for CA. The one without agents was set as negative control. After 6, 8, 12 and 24 h medication, the membrane was taken out, washed by PBS, cut up into tube containing 5 mL PBS for 20 min ultrasonic vibration. The pour-plate method was used to measure viable count. Each measurement was performed in triplicate.

Scanning electron microscope (SEM)

Amount of 2 mL TSB broth and 0.2 mL pathogen solution were loaded on 6-well plate containing sterile cover-slip to incubate for 1 day at 37℃. The cover-slip was taken out, rinsed by PBS, and placed into well containing medium with agent concentration of 2×MIC. The negative control had medium with no antimicrobial agent. The measures were repeated for three days. Then, the cover-slip was taken out and rinsed by PBS. After drying, the sample was fixed by 2.5% glutaraldehyde overnight, and dehydrated by 20%, 40%, 70% and 100% ethanol for morphological observation by SEM as described previously.[16]

Statistical analysis

Data were expressed as Mean±standard deviation (*SD*). The statistical analysis was performed by SPSS 11.0 (SPSS Co., Chicago, IL, USA) for Windows. The comparison among groups adopted *t*-test. Different was considered to be significant at $P<0.05$.

RESULTS

MICs of antimicrobial agents

According to the tests described above, the MICs of three antimicrobial agents, namely SH, AZM and FLU, for SE and CA were determined (Table 1).

Table 1 MICs of sodium houttuyfonate, azithromycin and fluconazole for Staphylococcus epidermidis and Candida albicans

Antimicrobial agents	MIC_{SE} (μg/mL)	MIC_{CA} (μg/mL)
SH	256	1024
AZM	64	-
FLU	-	2

Notes: SH: sodium houttuyfonate; AZM: azithromycin; FLU: fluconazole; MICs: Minimal inhibitory concentrations; MIC_{SE}: minimal inhibitory concentrations of antimicrobial agent for Staphylococcus epidermidis; MIC_{CA}: minimal inhibitory concentrations of antimicrobial agent for Candida albicans.

Suppressions of antimicrobial agents on biofilm mass and attachment

Figure 1 showed better antimicrobial effect of SH on the biofilm formation of SE compared with that of AZM, as most results had statistical significance ($P<0.01$ or $P=0.000$). After the 1st, 2nd and 3rd day of medications, the suppressions of biofilm were about 60% ($P<0.01$), 76% ($P=0.000$) and 75% ($P=0.000$) by 2×MIC SH, and about 17%, 65% ($P<0.01$) and 54% ($P<0.05$) by 2×MIC AZM. Figure 2 also showed the superior antifungal effect of SH to FLU, as the suppressions of biofilm were about 90% ($P=0.000$), 88% ($P=0.000$) and 90% ($P=0.000$) by 2×MIC SH, and about 92% ($P=0.000$), 58% ($P=0.000$) and 75% ($P=0.000$) by 2×MIC FLU after the 1st, 2nd and 3rd day of medications. Figure 3 revealed that the inhibitions of biofilm attachment had no significant difference for SE by SH and AZM and CA by SH and FLU, although the bacterial colonies were suppressed largely by either traditional antibiotics (i.e. AZM and FLU) or SH.

Morphological observation by SEM

Biofilm of SE (Figure 4) disappeared and hyphae of CA (Figure 5) vanished by 2×MIC AZM and FLU, respectively. SH, however, presented more powerful inhibition on SE and CA than AZM and FLU did, as pathogenic cells largely decreased.

DISCUSSION

SE from prokaryotic bacteria and CA from eukaryotic fungi are both opportunistically lethal to immunosuppressed and microflora-disordered patients with biofilm formation as one of the underpinnings of their infection repetitions, and both pathogens have been often found to be coexisted in topical focus. Therefore, more efforts have been concentrated on the search of effective antimicrobial agents, especially with favorable inhibitions on both certain kinds of bacteria and fungi. Benefited from vast inherited prescriptions and medicinal materials which could be dating back to hundreds of years ago, traditional Chinese herbal medicines have been considered the promising candidate, e.g. SH. SH has been realized to have mild anti-biofilm treatment on SA,[17] and could play a synergistic role in biofilm suppression of PA, both of which were also common opportunistic pathogens in nosocomial infections.[13] Firstly in our experiments, it was proved that SH had robust antipathogenic effects on either SE or CA which is termed as a dimorphic fungus with either a yeast form or a hyphal form, and SH could control the morphological switch which contributes to the virulence of CA.[18]

Generally, the traditional Chinese herbal medicine in purified form often showed more moderate or higher MIC against pathogen biofilms compared to the traditional antibiotics or western antifungals. Interestingly in this work, we found that SH seemed to be more efficient than AZM and FLU on biofilm inhibitions of SE and CA, and it exerted more suppression on CA than SE under the same concentration of 2×MIC. It could be explained possibly from two aspects: first, the biofilm development can be generally divided into three phases at least, namely attachment, mature, and dispersion either from bacterial or fungal aspects, and the attachment phase determines the subsequent bioflim maturation. In terms of our results, the initial attachment suppression of SE or CA might be one of the leading cause for SH antimicrobial effects, as SH could inhibit either SE or CA attachment even after 6 h medication. Second, the biofilm pathogens, as known, were usually enclosed by extracellular polymer substances

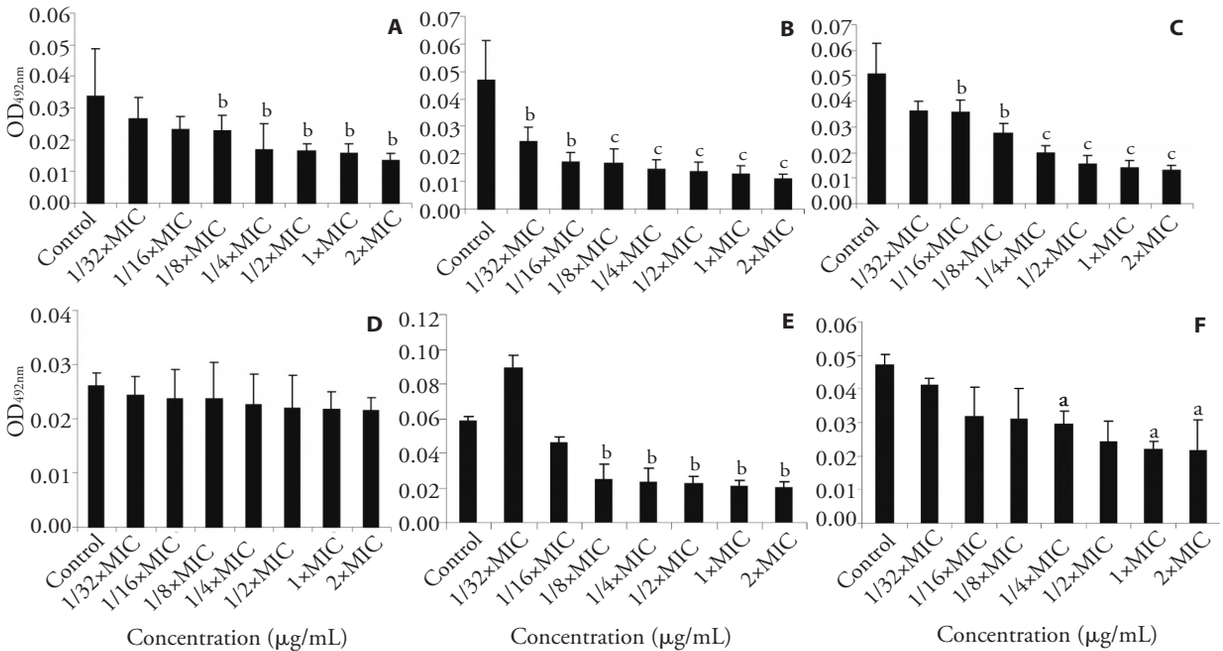

Figure 1 Effects of sodium houttuyfonate and azithromycin at different concentrations on biofilm growth of Staphylococcus epidermidis after days 1, 2 and 3

The suppressions on biofilm growth of Staphylococcus epidermidis after days 1 (A), 2 (B) and 3 (C) of sodium houttuyfonate medication and after days 1 (D), 2 (E) and 3 (F) of azithromycin medication with respective agent concentrations of 1/32×MIC, 1/16×MIC, 1/8×MIC, 1/4×MIC, 1/2×MIC, 1×MIC and 2×MIC. MIC: minimal inhibitory concentration. The control had only trypticase soy broth medium with no agents. The statistical significances of all data are reported to be compared with the control group. [a]*P*< 0.01; [b]*P*=0.000; [c]*P*<0.05. *n*=3. MICs: minimal inhibitory concentrations.

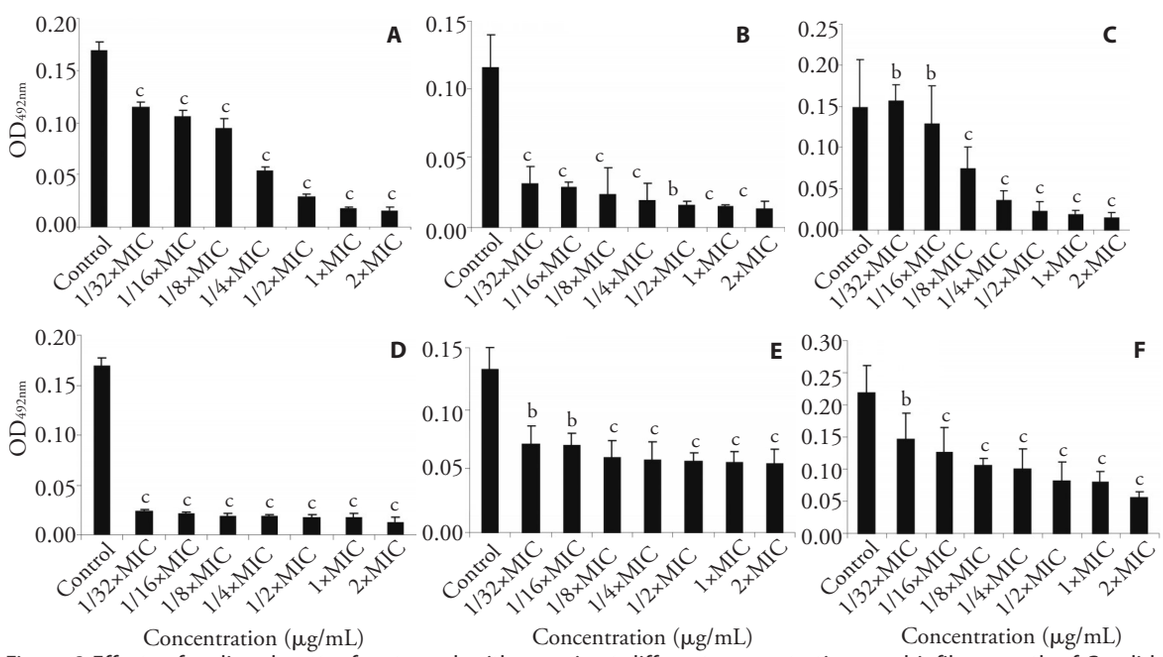

Figure 2 Effects of sodium houttuyfonate and azithromycin at different concentrations on biofilm growth of Candida albicans after days 1, 2 and 3

The suppressions on biofilm growth of Candida albicans after (A) 1 day, (B) 2 days and (C) 3 days of sodium houttuyfonate medication and after (D) 1 day, (E) 2 days and (F) 3 days of fluconazole medication with agent concentrations of 1/32×MIC, 1/16×MIC, 1/8×MIC, 1/4×MIC, 1/2×MIC, 1×MIC and 2×MIC. The control had only trypticase soy broth medium with no agents. The statistical significance of all data is reported to be compared with the control group. [a]*P*=0.000; [b]*P*<0.01. *n*=3. MICs: minimal inhibitory concentrations.

(EPS) which were composed of many ingredients including proteins, polysaccharide, DNA, etc. In our experiments, SH seemed to be more inclined to remove the EPS than the traditional antibiotics, as pathogenic biomass had more reduction by SH than that by traditional ones with both having nearly the same pathogenic colonies. Nevertheless, there need more proofs to clarify the underlying suppression mechanism of SH on SE and CA biofilms, as the results presented here might be implicated with multiple factors, such as temperature, medium, strains, etc.

In summary, SH, as a traditional Chinese herb, can be

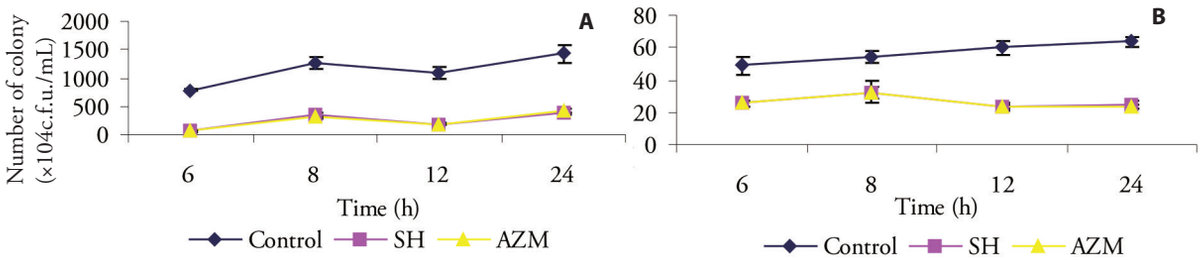

Figure 3 Number of colonies under different medications at 6, 8, 12 and 24 h
A: 2×MIC sodium houttuyfonate and 2×MIC azithromycin for Staphylococcus epidermidis; B: 2×MIC sodium houttuyfonate and 2×MIC fluconazole for Candida albicans. The control had only trypticase soy broth medium with no agents. SH: sodium houttuyfonate; AZM: azithromycin; FLU: fluconazole; MICs: minimal inhibitory concentrations.

Figure 4 Morphologies of Staphylococcus epidermidis under different medications on day 3 by scanning electron microscope
A: no drugs with only trypticase soy broth medium; B: 2×MIC azithromycin; C: 2×MIC sodium houttuyfonate. MICs: Minimal inhibitory concentrations.

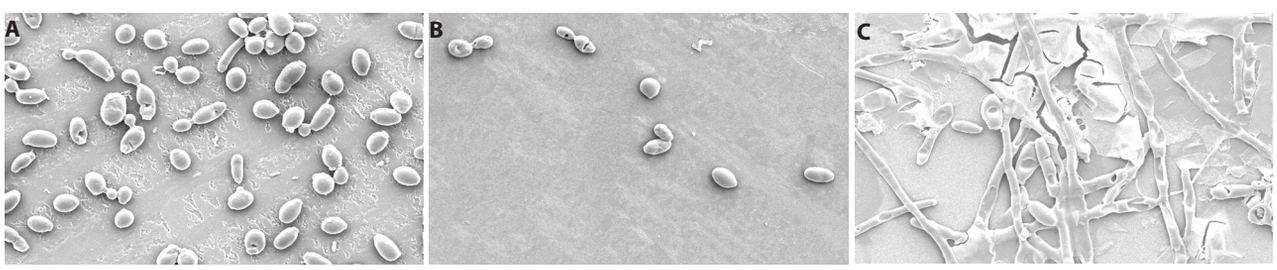

Figure 5 Morphologies of Candida albicans under different medications on day 3 by scanning electron microscope
A: no drugs with only trypticase soy broth medium; B: 2×MIC fluconazole; C: 2×MIC sodium houttuyfonate.

a promising potential for anti-biofilm research, especially in the anti-fungal field. And, subsequent *in vivo* tests of SH should be taken into consideration.

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